FINAL STUDY REPORT

STUDY TITLE

Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

Virus: Porcine Epidemic Diarrhea Virus

PRODUCT IDENTITY

H-31201 Lot 1310090416 and H-31202 Lot 1311251541

TEST GUIDELINE

OCSPP 810.2200

AUTHOR

Shanen Conway, B.S.
Study Director

STUDY COMPLETION DATE

May 30, 2014

PERFORMING LABORATORY

ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

SPONSOR

E.I. duPont de Nemours and Company
DuPont Experimental Station
Route 141 and Henry Clay, Building 402/5232B
Wilmington, DE 19803

SPONSOR STUDY NUMBER

DC&F-2014-001

PROJECT NUMBER

A16650

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality, on any basis whatsoever, is made for any information contained in this document. I acknowledge that information not designated as within the scope of FIFRA sec. 10(d)(1)(A), (B), or (C) and which pertains to a registered or previously registered pesticide is not entitled to confidential treatment and may be released to the public, subject to the provisions regarding disclosure to multinational entities under FIFRA 10(g).

Company: E.I. duPont de Nemours and Company

Company Agent: _____

_____
Title

_____ Date: _____
Signature

GOOD LABORATORY PRACTICE STATEMENT

The study referenced in this report was conducted in compliance with U.S. Environmental Protection Agency Good Laboratory Practice (GLP) Regulations set forth in 40 CFR Part 160.

The studies not performed by or under the direction of ATS Labs are exempt from this Good Laboratory Practice Statement and include: characterization and stability of the test substance(s).

Submitter:_____ Date:_____

Sponsor:_____ Date:_____

Study Director: Shanen L. Conway Date: 5/30/14
Shanen Conway, B.S.

QUALITY ASSURANCE UNIT SUMMARY

Study: Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

The objective of the Quality Assurance Unit is to monitor the conduct and reporting of non-clinical laboratory studies. These studies have been performed under Good Laboratory Practice Regulations (40 CFR Part 160) and in accordance to standard operating procedures and standard protocols. The Quality Assurance Unit maintains copies of study protocols and standard operating procedures and has inspected this study on the dates listed below. Studies are inspected at time intervals to assure the integrity of the study.

Phase Inspected	Date of Phase Inspection	Date Reported to Study Director	Date Reported to Management
Critical Phase Audit	May 12, 2014	May 12, 2014	May 13, 2014
Draft Report	May 27, 2014	May 27, 2014	May 27, 2014
Final Report	May 29, 2014	May 29, 2014	May 30, 2014

The findings of these inspections have been reported to Management and the Study Director.

Quality Assurance Auditor: Judy Heidemann Date: 5-30-14

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STUDY PERSONNEL

STUDY DIRECTOR: Shanen Conway, B.S.

Professional Personnel Involved:

Scott R. Steinagel, B.S.	- Director, Technical Operations
Katherine A. Paulson, M.L.T.	- Senior Virologist
Matthew Cantin, B.S.	- Lead Virologist
Erica Flinn, B.A.	- Associate Virologist
Nicole Nieland, B.S.	- Associate Virologist

STUDY REPORT

GENERAL STUDY INFORMATION

Study Title: Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

Project Number: A16650

Protocol Number: DUP01042514.PEDV

Sponsor:
E.I. duPont de Nemours and Company
DuPont Experimental Station
Route 141 and Henry Clay, Building 402/5232B
Wilmington, DE 19803

Testing Facility:
ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

TEST SUBSTANCE IDENTITY

Test Substance Name: Virkon®S H-31201 Lot 1310090416 and
Virkon®S H-31202 Lot 1311251541

Test Substance Characterization
Test substance characterization as to content, stability, solubility, storage, etc., (40 CFR, Part 160, Subpart F [160.105]) is the responsibility of the Sponsor.

STUDY DATES

Date Sample Received: April 29, 2014
Study Initiation Date: May 7, 2014
Experimental Start Date: May 12, 2014 (Start time: 2:22 p.m.)
Experimental End Date: May 19, 2014 (End time: 5:50 a.m.)
Study Completion Date: May 30, 2014

OBJECTIVE

The objective of this study was to evaluate the virucidal efficacy of a test substance for registration of a product as a virucide. The test procedure was to simulate the way in which the product is intended to be used. This method is in compliance with the requirements of and may be submitted to the U.S. Environmental Protection Agency (EPA).

SUMMARY OF RESULTS

Test Substance:	Virkon®S H-31201 Lot 1310090416 and Virkon®S H-31202 Lot 1311251541
Dilutions:	1:200, defined as 1 g test substance + 200 mL 400 ppm AOAC Synthetic Hard Water 1:600, defined as 1 g test substance + 600 mL 400 ppm AOAC Synthetic Hard Water
Virus:	Porcine Epidemic Diarrhea Virus, Strain Colorado 2013 Isolate
Exposure Time:	10 minutes
Exposure Temperature:	5°C (5.0°C)
Organic Soil Load:	5% fetal bovine serum
Efficacy Result:	Two lots of Virkon®S (H-31201 Lot 1310090416 and H-31202 Lot 1311251541) met the performance requirements specified in the study protocol. The results indicate **complete inactivation** of Porcine Epidemic Diarrhea Virus under these test conditions as required by the U.S. EPA.

TEST SYSTEM

1. Virus

The Colorado 2013 Isolate strain of Porcine Epidemic Diarrhea Virus used for this study was obtained from the National Veterinary Services Labs (NVSL). The stock virus was prepared by collecting the supernatant culture fluid from 75-100% infected culture cells. The cells were disrupted and cell debris removed by centrifugation at approximately 2000 RPM for five minutes at approximately 4°C. The supernatant was removed, aliquoted, and the high titer stock virus was stored at ≤-70°C until the day of use. On the day of use, two aliquots of stock virus (ATS Labs Lot PED-42) were removed, thawed, combined and maintained at a refrigerated temperature until used in the assay. The stock virus culture was adjusted to contain 5% fetal bovine serum as the organic soil load. The stock virus tested demonstrated cytopathic effects (CPE) typical of Porcine Epidemic Diarrhea Virus on Vero 76 cells.

2. Indicator Cell Cultures

Cultures of Vero 76 cells were originally obtained from the American Type Culture Collection, Manassas, VA (ATCC CRL-1587). The cells were propagated by ATS Labs personnel. The cells were seeded into multiwell cell culture plates and maintained at 36-38°C in a humidified atmosphere of 5-7% CO_2. On the day of testing, the cells were observed as having proper cell integrity and confluency, and therefore, were acceptable for use in this study.

All cell culture documentation was retained for the cell cultures used in the assay with respect to source, passage number, growth characteristics, seeding densities and the general condition of the cells.

3. Test Medium

Test medium used in this study was Minimum Essential Medium (MEM) supplemented with 10 µg/mL gentamicin, 100 units/mL penicillin, 2.5 µg/mL amphotericin B, 10% (v/v) tryptose phosphate broth and 5 µg/mL TPCK-trypsin.

TEST METHOD

1. Preparation of Test Substance

Two lots of test substance, Virkon®S H-31201 Lot 1310090416 and Virkon®S H-31202 Lot 1311251541, were tested at 1:200 and 1:600 dilutions, defined as 1 g test substance + 200 mL 400 ppm AOAC Synthetic Hard Water (1.00 g product + 200.0 mL water) and defined as 1 g test substance + 600 mL 400 ppm AOAC Synthetic Hard Water (1.00 g product + 600.0 mL water) as requested by the Sponsor. The test substance was in solution as determined by visual observation and used on the day of preparation. The prepared test substance was equilibrated to the exposure temperature prior to use.

The 400 ppm AOAC Synthetic Hard Water was prepared using 12.0 mL of Solution I and 12.0 mL of Solution II. The total volume of hard water was brought to approximately 3 liters using sterile deionized water. The 400 ppm hard water was prepared, titrated (at 419 ppm) and used on the day of testing.

2. Preparation of Virus Films

Films of virus were prepared by spreading 200 µL of virus inoculum uniformly over the bottoms of five separate 100 x 15 mm sterile glass petri dishes (without touching the sides of the petri dish). The virus films were dried at 20.0°C in a relative humidity of 30% until visibly dry (20 minutes).

3. Preparation of Sephadex Gel Filtration Columns

To reduce the cytotoxic level of the virus-test substance mixture prior to assay of virus, and/or to reduce the virucidal level of the test substance, virus was separated from the test substance by filtration through Sephadex LH-20 gel. On the day of testing, Sephadex columns were prepared by centrifuging the prepared Sephadex gel in sterile syringes for three minutes to clear the void volume. The columns were then ready to be used in the assay.

4. Input Virus Control (TABLE 1)
On the day of testing, the stock virus utilized in the assay was titered by 10-fold serial dilution and assayed for infectivity to determine the starting titer of the virus. The results of this control are for informational purposes only.

5. Treatment of Virus Films with the Test Substance (TABLE 2)
For each lot and each concentration of test substance, one dried virus film was individually exposed to a 2.00 mL aliquot of the use dilution of the test substance and held covered for 10 minutes at 5°C (5.0°C). The virus films were completely covered with the test substance. Just prior to the end of the exposure time, the plates were individually scraped with a cell scraper to resuspend the contents and at the end of the exposure time the virus-test substance mixtures were immediately passed through individual Sephadex columns utilizing the syringe plungers in order to detoxify the mixtures. The filtrates (10^{-1} dilution) were then titered by 10-fold serial dilution and assayed for infectivity and/or cytotoxicity.

6. Treatment of Dried Virus Control Film (TABLE 1)
One virus film was prepared as previously described (paragraph 2). The virus control film was exposed to 2.00 mL of test medium in lieu of the test substance and held covered for 10 minutes at 5°C (5.0°C). Just prior to the end of the exposure time, the virus control was scraped with a cell scraper and at the end of the exposure time the virus mixture was immediately passed through a Sephadex column in the same manner as the test virus (paragraph 5). The filtrate (10^{-1} dilution) was then titered by 10-fold serial dilution and assayed for infectivity.

7. Cytotoxicity Controls (TABLE 3)
A 2.00 mL aliquot of the use dilution of each concentration of each lot of the test substance was filtered through a Sephadex column and the filtrate was diluted serially in medium and inoculated into Vero 76 cell cultures. Cytotoxicity of the Vero 76 cell cultures was scored at the same time as the virus-test substance and virus control cultures.

8. Assay of Non-Virucidal Level of Test Substance (Neutralization Control) (TABLE 4)
Each dilution of the neutralized test substance (cytotoxicity control dilutions) was challenged with an aliquot of low titer stock virus to determine the dilution(s) of test substance at which virucidal activity, if any, was retained. Dilutions that showed virucidal activity were not considered in determining reduction of the virus by the test substance.

Using the cytotoxicity control dilutions prepared above, an additional set of indicator cell cultures was inoculated with a 200 µL aliquot of each dilution in quadruplicate. A 100 µL aliquot of low titer stock virus was inoculated into each cell culture well and the indicator cell cultures were incubated along with the test and virus control plates.

9. Infectivity Assays
The Vero 76 cell line, which exhibits cytopathic effect (CPE) in the presence of Porcine Epidemic Diarrhea Virus, was used as the indicator cell line in the infectivity assays. Cells in multiwell culture dishes were inoculated in quadruplicate with 200 µL of the dilutions prepared from test and control groups. The input virus control was inoculated in duplicate. Uninfected indicator cell cultures (cell controls) were inoculated with test medium alone. The cultures were incubated at 36-38°C in a humidified atmosphere of 5-7% CO_2 in sterile disposable cell culture labware. The cultures were scored periodically for seven days for the absence or presence of CPE, cytotoxicity, and for viability.

10. Statistical Methods: Not applicable

PROTOCOL CHANGES

Protocol Amendments:
No protocol amendments were required for this study.

Protocol Deviations:
No protocol deviations occurred during this study.

DATA ANALYSIS

Calculation of Titers

Viral and cytotoxicity titers are expressed as $-\log_{10}$ of the 50 percent titration endpoint for infectivity ($TCID_{50}$) or cytotoxicity (TCD_{50}), respectively, as calculated by the method of Spearman Karber.

$$\text{- Log of 1st dilution inoculated} - \left[\left(\left(\frac{\text{Sum of \% mortality at each dilution}}{100}\right) - 0.5\right) \times (\text{logarithm of dilution})\right]$$

Calculation of Log Reduction

Dried Virus Control Log_{10} $TCID_{50}$ – Test Substance Log_{10} $TCID_{50}$ = Log Reduction

STUDY ACCEPTANCE CRITERIA

U.S. EPA Submission

A valid test requires 1) that at least 4 log_{10} of infectivity be recovered from the dried virus control film; 2) that when cytotoxicity is evident, at least a 3-log reduction in titer is demonstrated beyond the cytotoxic level; 3) that the cell controls be negative for infectivity. **Note:** An efficacious product must demonstrate complete inactivation of the virus at all dilutions.

RECORD RETENTION

Study Specific Documents

All of the original raw data developed exclusively for this study shall be archived at ATS Labs, 1285 Corporate Center Drive, Suite 110, Eagan, MN 55121. These original data include, but are not limited to, the following:

1. All handwritten raw data for control and test substances including, but not limited to, notebooks, data forms and calculations.
2. Any protocol amendments/deviation notifications.
3. All measured data used in formulating the final report.
4. Memoranda, specifications, and other study specific correspondence relating to interpretation and evaluation of data, other than those documents contained in the final study report.
5. Original signed protocol.
6. Certified copy of the final study report.
7. Study-specific SOP deviations made during the study.

Test Substance Retention

The test substance will be discarded following study completion per Sponsor approved protocol. It is the responsibility of the Sponsor to retain a sample of the test substance.

REFERENCES

1. Annual Book of ASTM Standards, Section 11 Water and Environmental Technology Volume 11.05 Pesticides; Environmental Assessment; Hazardous Substances and Oil Spill Response, E 1053-11.
2. Annual Book of ASTM Standards, Section 11 Water and Environmental Technology Volume 11.05 Pesticides; Environmental Assessment; Hazardous Substances and Oil Spill Response, E 1482-12.
3. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, OCSPP 810.2000: General Considerations for Uses of Antimicrobial Agents, September 4, 2012.
4. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, OCSPP 810.2200: Disinfectants for Use on Hard Surfaces- Efficacy Data Recommendations, September 4, 2012.
5. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Lennette, E.H., Lennette, D.A. and Lennette, E.T. editors. Seventh edition, 1995.
6. Blackwell, J.H., and J.H.S. Chen. 1970. Effects of various germicidal chemicals on HEP-2 cell culture and Herpes simplex virus. J. AOAC 53:1229-1236.

STUDY RESULTS

Results of tests with two lots of test substance, Virkon®S H-31201 Lot 1310090416 and Virkon®S H-31202 Lot 1311251541, diluted 1:200 and 1:600, defined as 1 g test substance + 200 mL 400 ppm AOAC Synthetic Hard Water and defined as 1 g test substance + 600 mL 400 ppm AOAC Synthetic Hard Water, exposed to Porcine Epidemic Diarrhea Virus in the presence of a 5% fetal bovine serum organic soil load at 5°C (5.0°C) for 10 minutes are shown in Tables 1-4. All cell controls were negative for test virus infectivity.

The titer of the input virus control was 5.50 $\log_{10}$. The titer of the dried virus control was 4.50 $\log_{10}$. Following exposure, test virus infectivity was not detected in the virus-test substance mixture for either concentration of either lot at any dilution tested ($\leq$0.50 $\log_{10}$). Test substance cytotoxicity was not observed in either concentration of either lot at any dilution tested ($\leq$0.50 $\log_{10}$). The neutralization control (non-virucidal level of the test substance) indicates that the test substance was neutralized at $\leq$0.50 $\log_{10}$ for both concentrations of both lots. Taking the cytotoxicity and neutralization control results into consideration, the reduction in viral titer was $\geq$4.00 $\log_{10}$ for both concentrations of both lots.

STUDY CONCLUSION

Under the conditions of this investigation and in the presence of a 5% fetal bovine serum organic soil load, Virkon®S H-31201 Lot 1310090416 and Virkon®S H-31202 Lot 1311251541, diluted 1:200 and 1:600, defined as 1 g test substance + 200 mL 400 ppm AOAC Synthetic Hard Water and defined as 1 g test substance + 600 mL 400 ppm AOAC Synthetic Hard Water, demonstrated complete inactivation of Porcine Epidemic Diarrhea Virus following a 10 minute exposure time at 5°C (5.0°C) as required by the U.S. EPA.

In the opinion of the Study Director, there were no circumstances that may have adversely affected the quality or integrity of the data.

TABLE 1: Virus Controls Results

Dilution	Input Virus Control	Dried Virus Control
Cell Control	0 0	0 0 0 0
10^{-1}	+ +	+ + + +
10^{-2}	+ +	+ + + +
10^{-3}	+ +	+ + + +
10^{-4}	+ +	+ + + +
10^{-5}	+ +	0 0 0 0
10^{-6}	0 0	0 0 0 0
10^{-7}	0 0	NT
$TCID_{50}$/200 µL	$10^{5.50}$	$10^{4.50}$

(+) = Positive for the presence of test virus
(0) = No test virus recovered and/or no cytotoxicity present
(NT) = Not tested

TABLE 2: Test Results

Effects of Virkon®S H-31201 Lot 1310090416 and Virkon®S H-31202 Lot 1311251541 Following a 10 Minute Exposure to Porcine Epidemic Diarrhea Virus Dried on an Inanimate Surface

Dilution	Porcine Epidemic Diarrhea Virus + H-31201 Lot 1310090416 1:200 Dilution	Porcine Epidemic Diarrhea Virus + H-31201 Lot 1310090416 1:600 Dilution	Porcine Epidemic Diarrhea Virus + H-31202 Lot 1311251541 1:200 Dilution	Porcine Epidemic Diarrhea Virus + H-31202 Lot 1311251541 1:600 Dilution
Cell Control	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-1}	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-2}	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-3}	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-4}	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-5}	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-6}	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
$TCID_{50}$/200 µL	$\leq 10^{0.50}$	$\leq 10^{0.50}$	$\leq 10^{0.50}$	$\leq 10^{0.50}$

(0) = No test virus recovered and/or no cytotoxicity present

TABLE 3: Cytotoxicity Control Results

Cytotoxicity of Virkon®S H-31201 Lot 1310090416 and Virkon®S H-31202 Lot 1311251541 on Vero 76 Cell Cultures

Dilution	Porcine Epidemic Diarrhea Virus + H-31201 Lot 1310090416 1:200 Dilution	Porcine Epidemic Diarrhea Virus + H-31201 Lot 1310090416 1:600 Dilution	Porcine Epidemic Diarrhea Virus + H-31202 Lot 1311251541 1:200 Dilution	Porcine Epidemic Diarrhea Virus + H-31202 Lot 1311251541 1:600 Dilution
Cell Control	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-1}	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-2}	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-3}	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-4}	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-5}	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-6}	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
TCD_{50}/200 µL	$\leq 10^{0.50}$	$\leq 10^{0.50}$	$\leq 10^{0.50}$	$\leq 10^{0.50}$

(0) = No test virus recovered and/or no cytotoxicity present

TABLE 4: Neutralization Control Results

Non-Virucidal Level of the Test Substance (Neutralization Control)

Dilution	Porcine Epidemic Diarrhea Virus + H-31201 Lot 1310090416 1:200 Dilution	Porcine Epidemic Diarrhea Virus + H-31201 Lot 1310090416 1:600 Dilution	Porcine Epidemic Diarrhea Virus + H-31202 Lot 1311251541 1:200 Dilution	Porcine Epidemic Diarrhea Virus + H-31202 Lot 1311251541 1:600 Dilution
Cell Control	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-1}	+ + + +	+ + + +	+ + + +	+ + + +
10^{-2}	+ + + +	+ + + +	+ + + +	+ + + +
10^{-3}	+ + + +	+ + + +	+ + + +	+ + + +
10^{-4}	+ + + +	+ + + +	+ + + +	+ + + +
10^{-5}	+ + + +	+ + + +	+ + + +	+ + + +
10^{-6}	+ + + +	+ + + +	+ + + +	+ + + +
$TCID_{50}$/200 µL	$\leq 10^{0.50}$	$\leq 10^{0.50}$	$\leq 10^{0.50}$	$\leq 10^{0.50}$

(+) = Positive for the presence of test virus after low titer stock virus added (neutralization control)
(0) = No test virus recovered and/or no cytotoxicity present

Results of the non-virucidal level control indicate that the test substance was neutralized at a $TCID_{50}$/200 µL of ≤ 0.50 $\log_{10}$ for both concentrations of both lots.

(For Laboratory Use Only)
ATS Labs Project # A16650

Scc 5-9-14

ATS LABS

PROTOCOL

Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

Virus: Porcine Epidemic Diarrhea Virus

PROTOCOL NUMBER

DUP01042514.PEDV

PREPARED FOR

E.I. duPont de Nemours and Company
DuPont Experimental Station
Route 141 and Henry Clay, Building 402/5232B
Wilmington, DE 19803

PERFORMING LABORATORY

ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

DATE

April 25, 2014

EXACT COPY
INITIALS SL DATE 5/30/14

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Protocol Number: DUP01042514.PEDV E.I. duPont de Nemours and Company
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Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

SPONSOR: E.I. duPont de Nemours and Company
DuPont Experimental Station
Route 141 and Henry Clay, Building 402/5232B
Wilmington, DE 19803

TEST FACILITY: ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

PURPOSE

The purpose of this study is to evaluate the virucidal efficacy of a test substance for registration of a product as a virucide. The test procedure is to simulate the way in which the product is intended to be used. This method is in compliance with the requirements of and may be submitted to, one or more of the following agencies as indicated by the Sponsor: U.S. Environmental Protection Agency (EPA), Health Canada Therapeutic Products Directorate (TPD) and Australian Therapeutic Goods Administration (TGA).

TEST SUBSTANCE CHARACTERIZATION

Test substance characterization as to content, stability, solubility, storage, etc., (40 CFR, Part 160, Subpart **F** [160.105]) is the responsibility of the Sponsor. The test substance shall be characterized by the Sponsor prior to the experimental start date of this study. Pertinent information, which may affect the outcome of this study, shall be communicated in writing to the Study Director upon sample submission to ATS Labs.

SCHEDULING AND DISCLAIMER OF WARRANTY

Experimental start dates are generally scheduled on a first-come/first-serve basis once ATS Labs receives the Sponsor approved/completed protocol, signed fee schedule and corresponding test substance(s). Based on all required materials being received at this time, the proposed experimental start date is May 5, 2014. Verbal results may be given upon completion of the study with a written report to follow on the proposed completion date of June 2, 2014. To expedite scheduling, please be sure all required paperwork and test substance documentation is complete/accurate upon arrival at ATS Labs.

If a test must be repeated, or a portion of it, because of failure by ATS Labs to adhere to specified procedures, it will be repeated free of charge. If a test must be repeated, or a portion of it, due to failure of internal controls, it will be repeated free of charge. "Methods Development" fees shall be assessed, however, if the test substance and/or test system require modifications due to complexity and difficulty of testing.

If the Sponsor requests a repeat test, they will be charged for an additional test.

Neither the name of ATS Labs nor any of its employees are to be used in advertising or other promotion without written consent from ATS Labs.

The Sponsor is responsible for any rejection of the final report by the regulatory agency of its submission concerning report format, pagination, etc. To prevent rejection, Sponsor should carefully review the ATS Labs final report and notify ATS Labs of any perceived deficiencies in these areas before submission of the report to the regulatory agency. ATS Labs will make reasonable changes deemed necessary by the Sponsor, without altering the technical data.

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JUSTIFICATION FOR SELECTION OF THE TEST SYSTEM

Regulatory agencies require that a specific virucidal claim for a disinfectant intended for use on hard surfaces be supported by appropriate scientific data demonstrating the efficacy of the test substance against the claimed virus. Each agency will accept adequate data generated by any appropriate technique in support of a virucidal efficacy claim. This is accomplished by treating the target virus with the disinfectant (test substance) under conditions, which simulate as closely as possible, in the laboratory, the actual conditions under which the disinfectant is designed to be used. For disinfectant products intended for use on hard surfaces (dry, inanimate environmental surfaces), a carrier method is used in the generation of the supporting virological data. The Vero 76 cell line, which supports the growth of the Porcine Epidemic Diarrhea Virus, will be used in this study. The experimental design in this protocol meets these requirements.

TEST PRINCIPLE

A film of virus, dried on a glass surface, is exposed to the test substance for a specified exposure time. At the end of the exposure time, the virucidal and cytotoxic activities are removed from the virus-test substance mixture, and the mixture is assayed for viral infectivity by an accepted assay method. Appropriate virus, test substance cytotoxicity, and neutralization controls are run concurrently.

STUDY DESIGN

Dried virus films will be prepared in parallel and used as follows:

The appropriate number of films for each batch of test substance assayed per exposure time requested.

The appropriate number of films for virus control titration (titer of virus after drying) per exposure time requested.

At the end of the specified exposure time, resuspended virus-test substance mixtures will be detoxified and made non-virucidal by immediately adding the contents to a Sephadex gel filtration column followed by 10-fold serial dilutions in test medium. Each dilution is inoculated into indicator cell cultures. The resuspended virus control film and each batch of test substance alone will be treated in exactly the same manner. For analysis of infectivity, cultures will be held for the appropriate incubation period at the end of which time cultures will be scored for the presence of the test virus. Cultures will be monitored at that time for cell viability. Uninfected indicator cell cultures will be carried in parallel and similarly monitored. For analysis of cytotoxicity, the viability of cultures inoculated with dilutions of each test and cytotoxicity control will be determined. In addition to the above titrations for infectivity and cytotoxicity, the residual virucidal activity of the test substance after neutralization will be determined by adding a low titer of stock virus to each dilution of the test substance (cytotoxicity control dilutions). The resulting mixtures of dilutions are assayed for infectivity in order to determine the dilution(s) of test substance at which virucidal activity, if any, is retained.

VIRUS

The Colorado 2013 Isolate strain of Porcine Epidemic Diarrhea Virus to be used for this study was obtained from the National Veterinary Services Labs (NVSL). Stock virus is prepared by collecting the supernatant culture fluid from 75-100% infected culture cells. The cells are disrupted and cell debris removed by centrifugation. The supernatant is removed, aliquoted, and the high titer stock virus may be stored at ≤ -70°C until the day of use. Alternate methods of viral propagation may be utilized based on the growth requirements of the virus. The propagation method will be specified in the raw data and in the report. On the day of use the appropriate number of aliquots are removed, thawed, combined (if applicable) and maintained at a refrigerated temperature until used in the assay. **Note:** If the Sponsor requests an organic soil load challenge, fetal bovine serum (FBS) or the requested organic soil will be incorporated into the stock virus aliquot. The stock virus aliquot will be adjusted to yield the percent organic soil load requested.

INDICATOR CELL CULTURES

Cultures of Vero 76 cells were originally obtained from the American Type Culture Collection, Manassas, VA (ATCC CRL-1587). The cells are propagated by ATS Labs personnel. The cells are seeded into multiwell cell culture plates and maintained at 36-38°C in a humidified atmosphere of 5-7% CO_2. The confluency of the cells will be appropriate for the test virus. Vero 76 cells obtained from an alternate, reputable source may be used. The source of the cells will be specified in the final report. Alternatively Vero cells may be used in place of Vero 76 cells. The specific cell line used in the study will be documented in the raw data and reported.

All cell culture documentation is retained for the cell cultures used in this assay with respect to source, passage number, growth characteristics, seeding densities and the general condition of the cells.

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TEST MEDIUM

The test medium used for this assay is Minimum Essential Medium (MEM) supplemented with 0-10% (v/v) heat inactivated fetal bovine serum. The medium may also be supplemented with one or more of the following: 10 µg/mL gentamicin, 100 units/mL penicillin, 2.5 µg/mL amphotericin B, 1.0-2.0 mM L-glutamine, and 0.5 – 5 µg/mL trypsin. The composition of the test medium may be altered based on the virus and/or cells. The composition of the medium will be specified in the raw data and in the report.

PREPARATION OF TEST SUBSTANCE

The dilution of test substance(s) will be used as recommended by the Sponsor. The product will be pre-equilibrated to the desired test temperature if applicable.

PREPARATION OF VIRUS FILMS

Films of virus will be prepared by spreading 200 µL of virus inoculum uniformly over the bottom of the appropriate number of 100 X 15 mm sterile glass petri dishes (without touching the sides of the petri dish). The virus will be air-dried at 10°C-30°C until visibly dry (≥20 minutes). A calibrated timer will be used for timing the drying. The drying conditions (temperature and humidity) will be appropriate for the test virus for the purpose of obtaining maximum survival following drying. The actual drying conditions, drying time and calibrated timer used will be clearly documented.

For U. S. EPA, Australian TGA, and internal/other use only, one dried virus film per batch of test substance will be assayed unless otherwise requested. For Health Canada TPD, five dried virus films per batch of test substance will be assayed unless otherwise requested. If multiple regulatory agencies are chosen, the greater number of virus films will be assayed.

TEST METHOD

Preparation of Sephadex Gel Filtration Columns

To reduce the cytotoxic level of the virus-test substance mixture prior to assay of virus, and/or to reduce the virucidal level of the test substance, virus is separated from the test substance by filtration through Sephadex gel. The type of Sephadex used will be specified in the final report. On the day of testing, Sephadex columns are prepared by centrifuging the prepared Sephadex gel in sterile syringes for three minutes to clear the void volume. The columns are now ready to be used in the assay.

Input Virus Control

On the day of testing, the stock virus utilized in the assay will be titered by 10-fold serial dilution and assayed for infectivity to determine the starting titer of the virus. The results of this control are for informational purposes only.

Treatment of Virus Films with the Test Substance

For each batch of test substance assayed, the appropriate number of dried virus films are individually exposed to a 2.0 mL aliquot of the use dilution of the test substance (liquid products), or to the amount of spray released under use conditions (spray products) and held covered for the specified exposure time(s) and temperature. A calibrated timer will be used for timing the exposure. The actual temperature will be recorded. Just prior to the end of the exposure time, the plates are individually scraped with a cell scraper to resuspend the contents and at the end of the exposure time the virus-test substance mixtures are immediately passed through individual Sephadex columns utilizing the syringe plunger in order to detoxify the mixture. The filtrate (10^{-1} dilution) is then titered by serial dilution and assayed for infectivity and/or cytotoxicity. To further aid in the removing of the cytotoxic effects of the test substance to the indicator cell cultures, individual dilutions may be passed through additional individual Sephadex columns.

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Treatment of Dried Virus Control Film
The appropriate number of virus films are prepared as described previously for each exposure time assayed. The virus control films are run in parallel to the test virus but a 2.0 mL aliquot of test medium is added in lieu of the test substance. The virus control films are held covered and exposed to the test medium for the same exposure time and at the same exposure temperature as the test films are exposed to the test substance. A calibrated timer will be used for timing the exposure and the actual temperature will be recorded. Just prior to the end of the exposure time, the virus films are individually scraped as previously described and at the end of the exposure time the mixtures are immediately passed through individual Sephadex columns utilizing the syringe plunger. The filtrate (10^{-1} dilution) is then titered by serial dilution and assayed for infectivity. If additional Sephadex columns were used to further reduce the cytotoxic effects in the test substance assay, the same dilutions of the virus control will be passed through additional individual Sephadex columns.

Cytotoxicity Control
A 2.0 mL aliquot of each batch of test substance (liquid products) or the amount of the test substance recovered when sprayed onto a sterile petri dish (spray products), is filtered through a Sephadex column utilizing the syringe plunger and the filtrate is diluted serially in medium and inoculated into cell cultures for assay of cytotoxicity concurrently with the virus control and test substance-treated virus samples. For spray products, the cytotoxicity control will be held covered for the longest requested exposure time at the requested exposure temperature. A calibrated timer will be used for timing the exposure. If additional Sephadex columns were used to further reduce the cytotoxic effects in the test substance assay, the same dilutions of the cytotoxicity control will be passed through additional individual Sephadex columns.

Assay of Non-Virucidal Level of Test Substance (Neutralization Control)
Each dilution of the neutralized test substance (cytotoxicity control dilutions) will be challenged with an aliquot of low titer stock virus to determine the dilution(s) of test substance at which virucidal activity, if any, is retained. Dilutions that show virucidal activity will not be considered in determining reduction of the virus by the test substance.

Using the cytotoxicity control dilutions prepared above, an additional set of indicator cell cultures will be inoculated with a ~~100 µL~~ * JCS 5/7/14 aliquot of each dilution in quadruplicate. A 100 µL aliquot of low titer stock virus will be inoculated into each cell culture well and the indicator cell cultures will be incubated along with the test and virus control plates. * 200 mL will be used instead of 100 mL. JCS/7/14
Correction to protocol. JCS/7/14

Infectivity Assays
The Vero 76 cell line, which exhibits cytopathic effect (CPE) in the presence of Porcine Epidemic Diarrhea Virus, will be used as the indicator cell line in the infectivity assays. Cells in multiwell culture dishes will be inoculated in quadruplicate with * ~~100 µL~~ JCS 5/7/14 of the dilutions prepared from test and control groups. The input virus control will be inoculated in duplicate. Uninfected indicator cell cultures (cell controls) will be inoculated with test medium alone. Cultures are incubated at 36-38°C in a humidified atmosphere of 5-7% CO_2 in sterile disposable cell culture labware. The cultures will be scored periodically for approximately seven days for the absence or presence of CPE, cytotoxicity and for viability.

DATA ANALYSIS

Calculation of Titers
Viral and cytotoxicity titers will be expressed as $-\log_{10}$ of the 50 percent titration endpoint for infectivity ($TCID_{50}$) or cytotoxicity (TCD_{50}), respectively, as calculated by the method of Spearman Karber.

$$-\text{Log of 1st dilution inoculated} - \left[\left(\left(\frac{\text{Sum of \% mortality at each dilution}}{100}\right) - 0.5\right) \times (\text{logarithm of dilution})\right]$$

Calculation of Log Reduction
Dried Virus Control Log_{10} $TCID_{50}$ – Test Substance Log_{10} $TCID_{50}$ = Log Reduction

If multiple dried virus control replicates are performed, the average titer of the replicates will be calculated and the average titer will be used to calculate the log reduction in viral titer of the individual test replicates.

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PROCEDURE FOR IDENTIFICATION OF THE TEST SYSTEM

The specialized virucidal testing section of ATS Labs maintains Standard Operating Procedures (SOPs) relative to virucidal efficacy testing studies. Virucidal efficacy testing is performed in strict adherence to these SOPs which have been constructed to cover all aspects of the work including, but not limited to, receipt, log-in, and tracking of biological reagents including virus and cell stocks for purposes of identification, receipt and use of chemical reagents including cell culture medium components, etc. These procedures are designed to document each step of virucidal efficacy testing studies. Appropriate references to medium, batch number, etc. are documented in the raw data collected during the course of each study.

Additionally, each virucidal efficacy test is assigned a unique Project Number when the Study Director initiates the protocol for the study. This number is used for identification of the test culture plates, etc. during the course of the test. Test culture plates are also labeled with reference to the test virus, experimental start date, and test product. These measures are designed to document the identity of the test system.

METHOD FOR CONTROL OF BIAS: N/A

STUDY ACCEPTANCE CRITERIA

Only the applicable acceptance criteria and references for the regulatory agency reviewing the data will be included in the final report.

U.S. EPA Submission

A valid test requires 1) that at least 4 $\log_{10}$ of infectivity be recovered from the dried virus control film; 2) that when cytotoxicity is evident, at least a 3-log reduction in titer is demonstrated beyond the cytotoxic level; 3) that the cell controls be negative for infectivity. If any of the previous requirements are not met, the test may be repeated under the current protocol number. **Note:** An efficacious product must demonstrate complete inactivation of the virus at all dilutions.

Health Canada TPD Submission

A valid test requires 1) at least a 4-log infectivity be recovered from the dried virus control film beyond the cytotoxic level of the test substance; 2) that the cell controls be negative for infectivity. If any of the previous requirements are not met, the test may be repeated under the current protocol number. **Note:** An efficacious product must demonstrate at least a 3 $\log_{10}$ reduction in viral titer beyond the cytotoxic level of the test substance.

Australian TGA Submission

A valid test requires 1) that at least 4 $\log_{10}$ of infectivity be recovered from the dried virus control film; 2) that when cytotoxicity is evident, at least a 3-log reduction in titer is demonstrated beyond the cytotoxic level; 3) that the cell controls be negative for infectivity. If any of the previous requirements are not met, the test may be repeated under the current protocol number. **Note:** An efficacious product must demonstrate complete inactivation of the virus at all dilutions.

FINAL REPORT

The report will include, but not be limited to, identification of the sample and date received, dates on which the test was initiated and completed, identification of the virus strain used and composition of the inoculum, description of cells, medium and reagents, description of the methods employed, tabulated results, calculated titers for infectivity and cytotoxicity, and a conclusion as it relates to the purpose of the test. A draft report may be requested by the Sponsor. The final report will be prepared once the Sponsor has reviewed the draft report and notified the Study Director to complete the study.

PROTOCOL CHANGES

If it becomes necessary to make changes in the approved protocol, the revision and reasons for change will be documented, reported to the Sponsor and will become a part of the permanent file for that study. Similarly, the Sponsor will be notified as soon as possible whenever an event occurs that may have an effect on the validity of the study.

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TEST SUBSTANCE RETENTION

Test substance retention shall be the responsibility of the Sponsor. Unused test substance will be **discarded** following study completion unless otherwise requested.

RECORD RETENTION

Study Specific Documents

All of the original raw data developed exclusively for this study shall be archived at ATS Labs. These original data include, but are not limited to, the following:

1. All handwritten raw data for control and test substances including, but not limited to, notebooks, data forms and calculations.
2. Any protocol amendments/deviation notifications.
3. All measured data used in formulating the final report.
4. Memoranda, specifications, and other study specific correspondence relating to interpretation and evaluation of data, other than those documents contained in the final study report.
5. Original signed protocol.
6. Certified copy of the final study report.
7. Study-specific SOP deviations made during the study.

Facility Specific Documents

The following records shall also be archived at ATS Labs. These documents include, but are not limited to, the following:

1. SOPs which pertain to the study conducted.
2. Non study-specific SOP deviations made during the course of this study, which may affect the results obtained during this study.
3. Methods which were used or referenced in the study conducted.
4. QA reports for each QA inspection with comments.
5. Facility Records: Temperature Logs (ambient, incubator, etc.), Instrument Logs, Calibration and Maintenance Records.
6. Current curriculum vitae, training records, and job descriptions for all personnel involved in the study.

PROPOSED STATISTICAL METHODS: N/A

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REFERENCES

1. Annual Book of ASTM Standards, Section 11 Water and Environmental Technology Volume 11.05 Pesticides; Environmental Assessment; Hazardous Substances and Oil Spill Response, E1053-11.
2. Annual Book of ASTM Standards, Section 11 Water and Environmental Technology Volume 11.05 Pesticides; Environmental Assessment; Hazardous Substances and Oil Spill Response, E1482-12.
3. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, OCSPP 810.2000: General Considerations for Uses of Antimicrobial Agents, September 4, 2012.
4. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, OCSPP 810.2200: Disinfectants for Use on Hard Surfaces – Efficacy Data Recommendations, September 4, 2012.
5. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Lennette, E.H., Lennette, D.A. and Lennette, E.T. editors. Seventh edition, 1995.
6. Blackwell, J.H., and J.H.S. Chen. 1970. Effects of various germicidal chemicals on HEP-2 cell culture and Herpes simplex virus. J. AOAC 53:1229-1236.
7. Canadian General Standards Board, Minister of Public Works and Government Services, August 1997. Assessment of Efficacy of Antimicrobial Agents for Use on Environmental Surfaces and Medical Devices, CAN/CGSB-2.161-97.
8. Health Canada Therapeutic Products Directorate, October 29, 2007. Guidance Document: Disinfectant Drugs, Health Products and Food Branch.
9. Australian Therapeutic Goods Administration (TGA), February 1998. Guidelines for the Evaluation of Sterilants and Disinfectants.
10. Australian Therapeutic Goods Administration (TGA), February 1998. Therapeutic Goods Order No. 54: Standard for Disinfectants and Sterilants.
11. Australian Therapeutic Goods Administration (TGA), March 1997. Therapeutic Goods Order No. 54A: Amendment to Standard for Disinfectants and Sterilants (TGO 54).
12. Australian Therapeutic Goods Administration (TGA), July 2005. Draft Guidelines for the Evaluation of Household/Commercial and Hospital Grade Disinfectants.

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STUDY INFORMATION

(All sections must be completed prior to submitting protocol)

Test Substance (Name and Batch Number - exactly as it should appear on final report):
H-31201 = Lot# 1310090416 H-31202 = Lot# 1311251541 Page 1
Testing at the lower certified limit (LCL) for the hardest-to-kill virus on your label is required for registration.
Page 7: Study Report Test Substance Name = Virkon S Lot/Batch = H-31201 = Lot 1310090416
H-31202 = Lot 1311251541

Product Description

- ☐ Quaternary ammonia
- ☐ Iodophor
- ☐ Peracetic acid
- ☐ Peroxide
- ☐ Sodium hypochlorite
- ☒ Other 21.4% potassium monopersulfate 1.5% Sodium Chloride 77.09% other ingredients

Test Substance Active Concentration (upon submission to ATS Labs):

Storage Conditions

- ☒ Room Temperature
- ☐ 2-8°C
- ☐ Other _____

Hazards

- ☐ None known: Use Standard Precautions
- ☒ Material Safety Data Sheet, Attached for each product
- ☐ As Follows: _____

Product Preparation

- ☐ No dilution required, Use as received (RTU)
- ☒ *Dilution(s) to be tested:

1:200 + 1:600 defined as 1 gram → 200ml + 600ml
(example: 1 oz/gallon) (amount of test substance) (amount of diluent)

- ☐ Deionized Water (Filter or Autoclave Sterilized)
- ☐ Tap Water (Filter or Autoclave Sterilized)
- ☒ AOAC Synthetic Hard Water: 400 PPM
- ☐ Other _____

Note: An equivalent dilution may be made unless otherwise requested by the Sponsor.

Test Virus: Porcine Epidemic Diarrhea Virus

Exposure Time: 10 minutes

Exposure Temperature:

- ☐ Room temperature (to be based on regulatory agency of submission)
- ☒ Other: 5 °C (please specify range)

Directions for application of aerosol/spray products: _____
☒ Check here if spray instructions are not applicable.

Organic Soil Load

- ☐ 1% fetal bovine serum (minimum level that can be tested)
- ☒ 5% fetal bovine serum
- ☐ Other _____

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REGULATORY AGENCY(S) THAT MAY REVIEW DATA

- ☑ U.S. EPA
- ☐ Health Canada (Canadian TPD)
- ☐ Therapeutic Goods Administration (Australian TGA)
- ☐ Not applicable - For internal/other use only (Efficacy result will be based on U.S. EPA requirements)

COMPLIANCE

This study will be conducted in compliance with the EPA Good Laboratory Practices Regulations of 40 CFR Part 160 (Federal Register Notice [August 17, 1989]) and in accordance to standard operating procedures.

☑ Yes
☐ No (Non-GLP Study)

PROTOCOL MODIFICATIONS

☐ Approved without modification
☑ Approved with modification

Not applicable, not being evaluated for Health Canada. JCS/7/14

If testing is performed for Health Canada submission, testing will be performed following the submission requirements of the US EPA. Reference numbers 11 and 12 are changed to: 11. Health Canada, January, 2014. Guidance Document - Disinfectant Drugs. 12. Health Canada, January, 2014. Guidance Document – Safety and Efficacy Requirements for Hard Surface Disinfectant Drugs.

JCS/7/14

JCS changes on page 5 in the Assay at Non-Virucidal Level of Test Substance section and Infectivity Assays section. JCS/7/14

PROTOCOL ATTACHMENTS

Supplemental Information Form Attached - ☐ Yes ☑ No

TEST SUBSTANCE SHIPMENT STATUS

☒ Has been used in one or more previous studies at ATS Labs.
☐ Has been shipped to ATS Labs (but has not been used in a previous study).
Date shipped to ATS Labs:_____ Sent via *overnight* delivery? ☐ Yes ☐ No
☐ Will be shipped to ATS Labs.
Date of expected receipt at ATS Labs:_____
☐ Sender (if other than Sponsor): Stacy Dixon
Product Manager Hacco Products
Neogen Corp.
1-800-477-8201

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APPROVAL SIGNATURES

SPONSOR:

NAME: Ms. Sherrill Nurnberg TITLE: Microbiology Associate Investigator

SIGNATURE: Sherrill Nurnberg DATE: 4/30/14

PHONE: (302) 695 - 8497 FAX: (302) 695 - 8680 EMAIL: Sherrill.Nurnberg@USA.dupont.com

For confidentiality purposes, study information will be released only to the sponsor/representative signing the protocol (above) unless other individuals are specifically authorized in writing to receive study information.

Other individuals authorized to receive information regarding this study: ☐ See Attached

ATS Labs: wrong location 5/7/14

NAME: ~~Marion J. Conway~~ Shanen Conway
Study Director

SIGNATURE: Shanen J. Conway DATE: 5/7/14
Study Director

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